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PERSPECTIVE

Active integration: new strategies for transgenesis

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Abstract This paper presents novel methods for producing transgenic animals, with a further emphasis on how these techniques may someday be applied in gene therapy. There are several passive methods for transgenesis, such as pronuclear microinjection (PNI) and Intracytoplasmic Sperm Injection-Mediated Transgenesis (ICSI-Tr), which rely on the repair mechanisms of the host for transgene (*tg*) insertion. ICSI-Tr has been shown to be an effective means of creating transgenic animals with a transfection efficiency of approximately 45% of animals born. Furthermore, because this involves the injection of the transgene into the cytoplasm of oocytes during fertilization, limited mosaicism has traditionally occurred using this technique. Current active transgenesis techniques involve the use of viruses, such as disarmed retroviruses which can insert genes into the host genome. However, these methods are limited by the size of the sequence that can be inserted, high embryo mortality, and randomness of insertion. A novel active method

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has been developed which combines ICSI-Tr with recombinases or transposases to increase transfection efficiency. This technique has been termed ''Active Transgenesis'' to imply that the *tg* is inserted into the host genome by enzymes supplied into the oocyte during *tg* introduction. DNA based methods alleviate many of the costs and time associated with purifying enzyme. Further studies have shown that RNA can be used for the transposase source. Using RNA may prevent problems with continued transposase activity that can occur if a DNA transposase is integrated into the host genome. At present *piggyBac* is the most effective transposon for stable integration in mammalian systems and as further studies are done to elucidate modifications which improve *piggyBac*'s specificity and efficacy, efficiency in creating transgenic animals should improve further. Subsequently, these methods may someday be used for gene therapy in humans.

Keywords Transposon · Transposase · Site-specific · Retrovirus · Recombinase

Introduction

The transgenic methods in use today were developed in the past 25 years and these traditional methods of genetic engineering and transgenesis insert genes at random locations within the large genome of higher organisms, resulting in loss of efficiency, unpredictable results, and unintended genetic consequences (Perry et al. 1999; Wall 2001; Lois et al. 2002; Wall 2002). The pronuclear microinjection technique was the first to be conceived and was developed specifically to produce germline transgenic mice. It has generated transgenic animals in a wide variety of mammalian species, usually with multiple concatemerised vector copies (Muller 1999; Wall 2001).

The most efficient transgenesis method to date is an active form of transgenesis which utilizes a Lentiviral technique first developed in rodents and later extended to farm animals (Lois et al. 2002; Hofmann et al. 2003). It makes use of disarmed retroviral vectors to actively insert desirable genes into the host organism, usually at the single celled embryo stage (Lois et al. 2002). However, there are several major drawbacks of this technique, such as the high embryo lethality (73%) and the relatively small amount of transgene (*tg*) DNA (9.5 kb) that can be transported, due to the limited physical volume of the viral particles (Lois et al. 2002). This, coupled with the required specialized containment facilities for retroviral production, make it prohibitive for many laboratories to exercise the retroviral transgenesis approach (Wall 2002). There are also concerns about the potential consequences of recombinant events between the viral vector and endogenous retroviruses, leading to the generation of new, more potent pathogenic agents.

Intracytoplasmic sperm injection-mediated transgenesis

The Institute for Biogenesis Research (IBR) at the University of Hawai'i in Manoa, developed another passive technique for the production of transgenic mice called Intracytoplasmic Sperm Injection-Mediated Transgenesis (ICSI-Tr) (Perry et al. 1999). During ICSI-Tr mouse spermatozoa are demembranated either by freeze-thawing or by treatment with TritonX-100, then incubated with linear, double stranded (ds) DNA that contains the *tg*. The rationale for this method was that the exposed perinuclear theca of the sperm head would interact with the DNA and act as a carrier for the *tg*. This sperm-DNA complex is then injected into oocytes by ICSI, and the *tg* is incorporated into the embryonic genome via the DNA repair mechanism (Perry 2000). During ICSI-Tr, the transfection efficiency is on average, 2.5% of oocytes injected (o_i) or 20% of animals born (a_b), with very little mosaicism (Perry et al. 1999). Recently a more efficient version of this method was reported where the efficiencies of o_i and a_b are 4.6% and 45%, respectively (Moreira et al. 2004). Both techniques, however, are examples of passive transgenesis procedures, which rely on the repair mechanism of the zygote for *tg* insertion. The ICSI-Tr techniques nevertheless have resulted in the insertion of *tg* in the region of >200 kb (Perry et al. 2001; Moreira et al. 2004; Osada et al. 2005).

To address some of the concerns noted above, a series of ICSI transgenesis methods have been

developed in which enzymes are used to facilitate the *tg* insertion. ICSI-Tr's reliance on the repair mechanisms of the zygote nucleus for the insertion of a *tg* limits the efficiency and specificity of this technique (Perry et al. 1999), and it has an efficiency of at best only 4.6% of oocytes injected resulting in transgenic mice (Moreira et al. 2004). To improve the efficiency of this method of transgenesis, an approach termed ''Active Transgenesis'' has been used. In this method, recombinases or transposases are injected into mouse oocytes to increase the efficiency of transgene integration into the genome. We have demonstrated that the bacterial recombinase protein RecA (Kaneko et al. 2005) and a mutated hyperactive Tn5 transposase protein (*Tn5p) (Suganuma et al. 2005) both increase transgenesis several fold as compared to conventional methods such as pronuclear microinjection (Nakanishi et al. 2002) and traditional ICSI-Tr (Perry et al. 1999).

Active transgenesis

The term ''Active Transgenesis'' was selected to imply that the *tg* is inserted into the host genome by enzymes supplied into the oocyte during *tg* introduction. Among approaches utilizing protein recombinases (RecA) (Kaneko et al. 2005) or transposases, the hyperactive Tn5 transposase protein (*Tn5p) was by far the most efficient method for introducing the *tg* in a transposon along with spermatozoa into unfertilized oocytes (TN:ICSI) (Suganuma et al. 2005). In our hands, this approach dramatically increased the efficiency of producing transgenic mice, with 11% of eggs injected and 22% of live births resulting in transmission of the *tg* DNA and over 75% of transgenic mice expressing the EGFP *tg*. Of these transgenic mice, 25% had one or two copies of the *tg* inserted in their genome. However, TN:ICSI methods suffer from cumbersome enzyme preparation techniques. A rich protein source is required, which in some cases is commercially available. DNA is much easier and faster to purify as compared to protein. Techniques to fractionate the source material must also not destroy the activity of the enzyme and an effective and sensitive assay for activity is required to check for purity. These factors contribute to the time and expense of enzymatic purification compared with DNA based procedures. Due to this, we have now moved away from the enzymatic insertions of *tg* and developed DNA and RNA based procedures that allow synthesis of the transposase in-situ.

We are now able to produce transgenic animals using active transgenesis with similar efficiencies as retroviral methods (Table 1). However, the F_0 mice produced appear mosaic, because transcription may occur after the one cell stage (Fig. 1). We might solve this by injecting cRNA to obtain transposase expression at the one cell embryo stage. Alternatively, epigenetic silencing could be occuring in certain tissues. We have also constructed transgenic ready mice containing the transposase under the control of the shortened Zona Pellucida 3 promoter (sZP3p), which would simplify ICSI-Tr and pronuclear techniques, by allowing transposase expression exclusively in the growing oocyte prior to the completion of the second meiotic division (Fig. 2).

There are potential hazards in using a DNA transposase for the integration of the *tg*. If the DNA transposase is stably integrated into the host DNA and expressed this could lead to remobilization of transposons and reintegration. The continued mobilization of the *tg* could lead to deleterious genomic modifications. A means of preventing this is through the use cRNA as the

Table 1 *piggyBac*:ICSI efficiency versus Lentiviral, pronuclear microinjection and ICSI-Tr

% of transgenic animals		
Delivery method	Oocytes injected (Oi)	Animals born (Ab)
Lentiviral Vectors	~20%	~80%
piggyBac:ICSI	~20%	~70%
Microinjection (PNI, ICSI-Tr)	~3–4.6%	~20–46%

The percent of oocytes which are successfully transfected using various delivery methods are shown (Oi). Also shown are the percentages of successfully transfected animals which are born (Ab) using various delivery methods. Transfection rates with PgB:ICSI are comparable to Lentiviral Vectors in both percentage of oocytes transfected and animals born

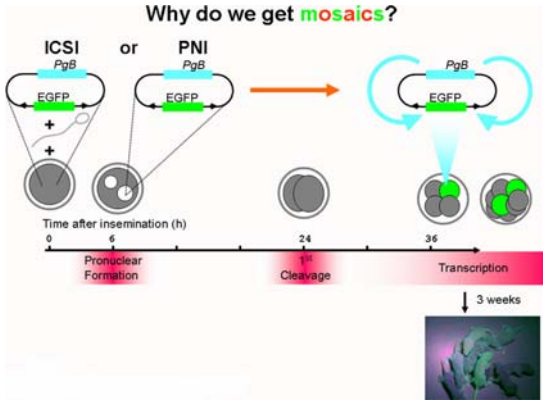

Fig. 1 All F_0 pups born are mosaics because transcription from the introduced *piggyBac* plasmid might not commence until after the first cell division. Therefore, for both ICSI and pronuclear microinjected embryos, only cells that have inherited the donor–helper plasmid express EGFP. Alternatively, epigenetic silencing could be occuring in certain tissues

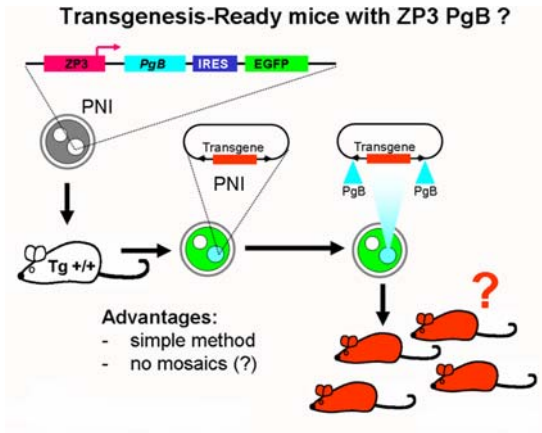

Fig. 2 The oocyte donor females are homozygous for the shortened ZP3 promoter driven bicistronic cassette. The sZP3 promoter is active only during oocyte development and oocytes transgenic for the *piggyBac* transposase are recognizable by EGFP expression. At metaphase II stage arrested oocytes are loaded with the *piggyBac* transposase protein and the transgene offered to them by the donor plasmid is readily inserted into the single cell embryo genome

source for the transposase enzymatic activity, which has been shown to be effective (Wilber et al. 2006). The transient nature of cRNA, limits the duration of transposase activity and would likely attenuate the risks of the integration of the transposase into the host genome. However, it is theoretically possible, although unlikely, that the RNA could undergo reverse transcription resulting in the possibility of non-homologous recombination into the host genome.

To determine which DNA transposase encoding plasmids may have the greatest affect on *tg* insertion, four commonly used transposon transfection systems were studied in four different mammalian cell lines (Wu et al. 2006), three of which were human. These initial experiments performed with the two plasmid system (Donor and Helper plasmids) demonstrated that *piggyBac* (*PB*), a transposase isolated from the cabbage looper moth *Trichoplusia ni*, and found to exhibit activity in a variety of species ranging from planarian to mammalian cells (Lobo et al. 2006), is the most effective mediator for stable insertion of *tg's* in all cell lines tested. One potential limitation of transposases is that instead of reaching a plateau in transposition with increasing transposase, transposon integration declines due to overproduction inhibition. We have observed overproduction inhibition with *PB* and it might also occur with *Tol2* if the ratio of helper to donor plasmid was increased (Wu et al. 2006). In contrast, Wilson and colleagues did not demonstrate overproduction inhibition with *PB* (Wilson et al. 2007). *PB* and *Tol2* have been found to be able to carry a larger cargo as compared to *Sleeping Beauty (SB)*. For example, the *PB* helper can be large (9.3–14.3 Kb) without significant reduction in transposition efficiency (Ding et al. 2005). Transposon systems have many attractive features as vectors for gene delivery, such as: (a) accommodating large *tg*; (b) being non-viral, they do not induce an immune response in rodent models; (c) inexpensive to mass produce (Kaminski and Summers 2003) and (d) mediating efficient *tg* integration which is stable and shows persistent expression (Ivics et al. 1997).

Mechanisms to improve specificity and efficiency of transfection

As *PB* is the most efficient transposon in mammalian systems (Wu et al. 2006), studies to modify *PB* to increase its specificity and transposition efficiency are in progress. Until recently the *PB* literature described the transposition machinery as a two-component system: a Helper plasmid

containing the transposase and a Donor plasmid containing the transposon (Wilson et al. 2007). We and recently others have simplified this approach by including the Helper and Donor components of *PB* in a single plasmid. This single plasmid approach makes it easier to effect transposition where if the plasmid has entered the nucleus of a cell, both components are included in it, likely facilitating transposition (Mikkelsen et al. 2003; Wilson et al. 2007) (Kaminski and Moisyadi, Unpublished). There is currently work being done with *PB* transposase to increase the transpositional efficiencies to that of retroviral vectors. There are methods to accomplish this goal such as PCR random mutagenesis or alanine substitutions utilizing mutagenic PCR primers (Goryshin and Reznikoff 1998; Yant et al. 2004; Pledger and Coates 2005; Keravala et al. 2006). One of us, Thomas Ryan, is pursuing *PB* active transgenesis in embryonic stem cells for the production of transgenic animals.

Other alterations include creating chimeric integrating enzymes for targeted integration. Chimeric transposons have significant advantages over site-specific retroviral vectors. For example, some transposases and other integrating enzymes (such as some serine recombinases) have a natural division into two domains (a catalytic domain that performs the DNA insertion, and a DNA directing domain which juxtaposes the integrating enzyme to the host DNA). Thus, some are likely amenable to chimeric approaches that swap the DNA-directing domain for one that targets the integrating enzyme to any chosen host DNA sequence. In certain integrating enzymes, e.g. transposases, the catalytic domains have little or no natural site specificity; therefore, it principally would be the engineered DNA binding domain that governs the site-specificity in chimeric integrating enzymes (Kaminski et al. 2002). Integrating enzymes such as from *SB*, *PB*, and/or φC31 have been widely used in plants, animals, insects, prokaryotes and frequently their usage is not limited to specific species (Coates et al. 2005; Kolb et al. 2005). The technology used to engineer the specific DNA binding domains is well defined, and has very flexible sequence specificity (Kolb et al. 2005). Transposases recognize loose consensus sequences, e.g. Tc1 superfamily integrate into TA dinucleotides, whereas site-specific recombinases recognize and mediate the recombination between short, well characterized DNA sequences resulting in the integration, excision or inversion of DNA fragments. Transposases have a theoretical advantage over recombinases in that they are potentially amenable to target any given region whereas recombinases are inherently limited to specific or closely related (pseudo) sites due to specific, larger sequence requirements of the catalytic domain. Thus, chimeric transposases could allow us to design vectors that would integrate into or around any site assuming the chromatin in that region is permissive for integration. Some recombinases, i.e. serine recombinases, allow unidirectional, irreversible integration and are limited to fewer sites, but can result in high frequencies of chromosomal rearrangements (Malla et al. 2005; Ehrhardt et al. 2006). We have tried to alter φC31 recombinase by coupling it to a DNA binding domain to target a specific pseudo-site but this resulted in loss of activity. In contrast, we have altered the *PB* transposase to direct integration and it has retained full activity (Wu et al. 2006).

Recent studies with *PB* and *Mos1* have shown promising results for targeted integration. A Gal4-*piggybac* and Gal4-*Mos1* chimera resulted in approximately a 11.6 and 12.7 fold increase, respectively, in targeted integration into a plasmid which contained a UAS tandem array (GAL4 binding site), presumably through tethering the transposon–transposase complex at the target site. There was a high level of specificity with the GAL4-Mos1 chimera with 51/53 recovered transposition events occurring at a single TA target site in the UAS plasmid and all but one were in the 5′-3′ orientation. In the Gal4-*piggyBac* chimera 45/67 inserted into a single TTAA target site and 36 of them were in the 5′-3′ orientation. These results suggest that the Gal4-UAS limits the number of target sites at which integration can occur, likely due to the tethering of the transposase close to the UAS target (Maragathavally et al. 2006). Mos1 does not appear to be functional in mammalian systems (Wu et al. 2006). However, the *PB* transposase coupled to the GAL4 DNA binding domain retains transposition activity similar to the wild-type, unlike Tol2 or

SB. Thus, we have recently constructed a transgenic mouse containing a genomic UAS tandem array and experiments are on-going to determine whether we preferentially target this region and if integration efficiencies are enhanced. We will also be testing the ability of a six-zinc finger domain to direct the integration of a transposon to an endogenous locus. The general strategy will be to direct integration to a gene that, when disrupted, will produce a phenotypic change for easy analysis but not adversely affect the health of the mouse. The tyrosinase (Tyr) gene, located on chromosome 7, would be one potential target. TYR is an enzyme present in melanocytes that catalyzes the production of melanin and other pigments from tyrosine by oxidation. Mutations in the Tyr gene result in albinism. In order to knock-out TYR function, a transposon gene trap vector with a splice acceptor site will be constructed so that it can integrate anywhere within the tyrosinase locus to create a knock-out of that gene's product. Since loss of pigmentation would not be anticipated to adversely affect the health of the mouse or the cell, this locus is considered a ''safe'' genomic location for testing targeted transposition.

Conclusions

By using transposons in conjunction with the passive technique ISCI-Tr, active transgenesis has been shown to greatly improve the rate and specificity of the insertion of *tg*. By providing the transposase (RNA, protein, or early expression from the ZP3) at the one cell stage, the rate of mosaicism should be reduced to allow for uniform expression of the *tg* throughout the host tissue. RNA has one major advantage, because the transposase source cannot integrate into the genome, thus transposase activity will always be transient. As further refinements are made to the transposases used, greater specificity and efficiency can be achieved. These techniques will reduce the cost, time, and unpredictability in the production of transgenic animals. Once there has been greater experience with creating transgenic animals these techniques might be used in human gene therapy.

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